NOCTURNAL HOMING IN THE AMBLYPYGID *PHRYNUS MARGINEMACULATUS*

Jacob M. Graving

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Committee:

Daniel D. Wiegmann, Advisor

Verner P. Bingman

Sheryl L. Coombs

ABSTRACT

Daniel D. Wiegmann, Advisor

Navigation has been studied rigorously in arthropods and the rich diversity of solutions these animals have evolved for such tasks is well documented. Nevertheless, these studies have focused on only a few diurnal species that live in simple environments with salient, reliable sources of spatial information. In this study, we examine the homing abilities of *Phrynus marginemaculatus*, a species of amblypygid (Class Arachnida, Order Amblypygi). Amblypygids are a bizarre, understudied order of arachnids that are able to navigate to a preferred shelter at night in challenging environments. The sensory and behavioral mechanisms by which amblypygids navigate are still unclear. To investigate this, we placed *P. marginemaculatus* individuals into an arena that contained a point odor source and two shelters that differed in quality. We monitored their homing behavior over a session of five to seven contiguous nights using an automated video tracker that continually recorded the coordinates of a subject in the arena. We found that individuals reliably exited and returned to a shelter each night and learned to discriminate between shelters without the use of visual information. Behavioral measurements from our coordinate data also provide insight into potential sensory and behavioral strategies this species uses while homing.

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INTRODUCTION

Navigation has been studied rigorously in a number of arthropods and the diversity of solutions to navigation problems that these animals have evolved is well documented (Cheng and Freas, 2015; Cheng, 2013; Collett et al., 2013; Collett and Collett, 2006; Collett and Graham, 2004; Dyer, 1998; Gould, 1998; Papi, 1992). Previous research has focused, however, on a few species of mostly diurnal, visually oriented animals (Perry et al., 2013; Dyer, 1998). The animals chosen for these studies also tend to inhabit two-dimensional environments with salient, reliable sensory cues, environments in which unimodal information (i.e., the information provided by a single sensory modality) and path integration (i.e., the estimation of position using self-generated, idiothetic movement cues) may be adequate to accomplish navigational tasks. Thus, it is unsurprising that relationships between behavioral, cognitive, and neural processes in complex navigational tasks remain cryptic (Kennedy and Norman, 2005).

More recent research illustrates the importance of studying goal-oriented behavior in a multisensory context (Gepner et al., 2015; Buehlmann et al., 2012; Steck et al., 2011; Duistermars and Frye, 2010; Gomez-Marin et al., 2010; Gilbert and Kuenen, 2008). Steck et al. (2009, 2011) demonstrated that homing in the desert ant *Cataglyphis fortis*, once thought to be solely a visually-mediated behavior, is in fact mediated by olfactory information at close range to the goal (i.e., nest) and that the use of both visual and olfactory modalities enhances homing performance. Gomez-Marin et al. (2010) likewise found that odor tracking by flying *Drosophila,* previously considered to be a simple case of "follow your nose", is in reality a complex, multisensory behavior involving both olfactory and mechanosensory information. These are just two cases in which a more comprehensive approach has created a richer understanding of how animals use their nervous systems to make decisions and generate goal-directed behavior.

More importantly, a broader understanding of navigation behavior requires more than just knowledge of the sensory modalities involved. Fundamental aspects of how these behaviors are studied must be improved. Indeed, Perry et al. (2013) argue convincingly that past research in animal learning has several important shortcomings. They argue that (1) it is not comparative enough and focuses on a skewed range of species; (2) too many assumptions are made about behavioral complexity with little evidence of the underlying neural mechanisms to support them; (3) it is no longer adequate to simply describe what animals can do without assessing the mechanisms used to accomplish these tasks. Additionally, in order to truly understand behavior, we must be able to measure its complexity quantitatively and accurately (Anderson and Perona, 2014). In recent years, the popularity of open-source programming languages has given behavioral scientists easy access to tools ideal for automated image-based measurement of behavior (Anderson and Perona, 2014; Dell et al., 2013). In this study we apply these tools for the automated tracking and quantification of homing behavior in a group of nocturnal arthropods called amblypygids. We use these tools to provide a rich description of their homing abilities in the laboratory and to derive insight into the sensory and behavioral mechanisms these animals use to accomplish a spatial learning task.

The order Amblypygi (Class Arachnida), known colloquially as “whip spiders” or “tailless whip scorpions”, is a bizarre, understudied group of arthropods. Unlike most previously studied arthropods, amblypygids navigate at night in complex tropical forests (Hebets et al., 2014a,b). These animals typically occupy small crevices in tree bark or the underside of rocks, emerging at night in search of food and mating opportunities and returning to their refuge before dawn (Weygoldt, 2000; Hebets, 2002; Hebets et al., 2014a,b). Although evidence of homing in amblypygids was, until recently, considered to be anecdotal (Strausfeld, 2012, p. 375), studies of

Heterophrynus and *Phrynus* species demonstrate the high degree of site fidelity this group of animals exhibits (Beck and Görke, 1974; Weygoldt, 1977; Hebets, 2002). More recently, Hebets et al. (2014a,b) provided strong evidence of their homing abilities. Indeed, Hebets et al. (2014a) demonstrated that *Phrynus pseudoparvulus*, native to Costa Rica, are skilled navigators that do not rely on path integration. Their impressive homing abilities also appear to involve the use of multimodal sensory information (Hebets et al., 2014b).

The sensory and behavioral mechanisms by which amblypygids navigate have yet to be thoroughly vetted (Hebets et al., 2014a,b). Past research on the sensory abilities of amblypygids is focused largely on the role on the antenniform legs as a primary source of information (Fig. 1; reviewed in Santer and Hebets 2011; Hebets et al, 2014b). These sensory appendages can be extended multiple body lengths and are covered with thousands of individual sensory hairs (Foelix and Hebets, 2001) capable of detecting olfactory (Hebets and Chapman, 2000), mechanosensory (Santer and Hebets 2009), and other types of stimuli (reviewed in Santer and Hebets, 2011; Weygoldt, 2000). Indeed, sensory manipulations of a single individual by Beck and Görke (1974) and multiple individuals by Hebets et al. (2014b) suggest that the antenniform legs, specifically the olfactory sensilla, play a vital role in successful homing.

While it may seem rational to ignore the amblypygid visual system as vital to navigation due to its diminutive nature and unknown functionality (Strausfeld, 2012 pp. 371; Hebets et al., 2014b), Hebets et al. (2014b) have shown that vision may be involved. This is sensible when one considers that a multitude of animals with seemingly poor visual systems are known to exploit visual cues for orientation in even the dimmest of habitats (Warrant and Dacke, 2010). Moreover, a model system for studying complex, multisensory navigation behavior may be that of nocturnal arthropods because many of these animals are adapted to environments where

sensory information can be limited and altogether unreliable both spatially and temporally (Warrant and Dacke, 2010). These animals live in an environment in which the ability to simultaneously use, or transition between, different sensory modalities and behavioral strategies may be especially advantageous. We know, in fact, that some arthropods are able to orient to their goals and navigate under such conditions, but the exact cognitive and behavioral mechanisms of their abilities are still unknown (Warrant and Dacke, 2010). Additionally, our recent characterization of the visual system in *Phrynus marginemaculatus* shows that these amblypygids possess at least one pair of functional eyes capable of collecting information that may be useful for orientation and navigation (Graving et al., In prep.). Combined with evidence from Hebets et al. (2014b), this suggests that navigation in amblypygids could involve the use of multiple sensory modalities and that amblypygid navigation may be an ideal model for studying the mechanisms of multisensory integration.

Determining the mechanism(s) involved in successful amblypygid homing is a task that will require laboratory experiments in which sensory conditions are directly controlled. The first step in this process is to find a suitable model for laboratory experiments and assess its ability to navigate in an artificially constructed environment. Here our goal is just that: to determine if amblypygids reliably home in the laboratory and describe their activity patterns and behavior. The establishment of this experimental paradigm would then allow for the systematic study of the behavioral, sensory and neural mechanisms involved in homing under conditions in which available information can be manipulated and tightly controlled. Toward this long-term goal, the specific aims of this study were to (1) evaluate the site fidelity of *P. marginemaculatus*, a species of amblypygid native to the Florida Keys, USA and (2) provide the first detailed description of amblypygid homing behavior in a laboratory setting, including (a) a description of nocturnal

activity patterns, (b) an assessment of space use, and (c) a quantitative characterization of their outbound and inbound paths while homing to an artificially constructed shelter within an experimental arena.

MATERIALS AND METHODS

Experimental Animals

We chose *P. marginemaculatus* as our model for this study (Fig. 2A). This species experiences wider ranges of temperature and humidity in its natural habitat than its more tropical relatives, thus reducing the likelihood that variable temperature and humidity in the laboratory would adversely affect behavior. We purchased wild-caught *P. marginemaculatus* individuals from a commercial supplier (Ken The Bug Guy LLC.; kenthebugguy.com) and collected additional subjects at the National Key Deer Refuge (Big Pine Key, Florida, USA; USFWS Permit Number FFO4RFKD-2015-06). We kept the animals separately in plastic deli cups that had a soil substrate and cardboard egg carton shelters. Animals were fed live crickets and misted with reverse osmosis water three times per week. We used 12 unsexed adults for this study.

Overhead broad-spectrum fluorescent lights controlled by a timer lighted the room in which we kept the animals and conducted the experiments (12h:12h light:dark cycle; 15:00-03:00 dusk-dawn). We kept animals on this light cycle for several weeks before the study was conducted and used the same light cycle for the overhead lights throughout the experiment. The room ranged in temperature from 21-26ºC and 20-60% humidity.

Experimental Design

P. marginemaculatus were placed individually into an arena that contained a point odor source and two shelters that differed in quality. We monitored their activity and behavior over a session of five to seven contiguous nights using an automated video tracker that continually recorded the coordinates of a subject in the arena when it exited a shelter.

The Arenas

Schematics of the behavioral arenas we used for this study are shown in Fig. 2. The walls of our arenas were constructed of clear acrylic plastic, and the arenas had dimensions of 1 x 1 x

0.3-m (L x W x H) (Fig. 2B-C). The bottom of each arena was constructed from a single piece of opaque white acrylic plastic, which created visual contrast between a subject and the arena floor to enhance the detection of a subject by the video tracker. Two 5-watt, broad-spectrum halogen lights were attached to two walls of arenas to motivate the utilization of a shelter by subjects during daylight hours. Each arena was elevated 12-cm off the laboratory floor by four supports made of 12-cm (outer diameter) PVC pipe placed underneath the arena near each corner.

The two shelters were constructed from 12-cm (outer diameter) PVC pipe cut to a height of 3-cm (Fig. 2D). The top of each shelter was fitted with a circular piece of black opaque acrylic plastic (to block light) that was covered by a circular piece of white opaque plastic to facilitate detection of a subject by the video tracker if it walked on the top of the shelter. The bottom of the shelter was fitted with a circular piece of transparent acrylic plastic. Each shelter had a 3 x 1-cm (L x H) entrance located at the level of the arena floor, was lined with black Velcro on the interior walls for subjects to climb on and contained a cellulose sponge saturated with reverse osmosis water for humidity.

We placed the shelters in opposite (diagonal) corners of the arena, directly over the PVC pipes used to elevate the arena, with each shelter entrance directed toward the nearest corner. Shelters and supports for each arena were placed 10-cm from the nearest wall, such that the each shelter entrance was located approximately 11-cm from the nearest walls (Fig. 2E). The PVC supports under each shelter contained a 3-watt, broad-spectrum, high-power LED light pointed upward toward the transparent bottom of a shelter and turned on in daylight hours. The floor of the arena was sufficiently transparent to allow this light to pass into the shelters. The amount of LED light that was transmitted into shelters determined the quality of a shelter. The light under

one shelter—the low-quality shelter (LQ)—was covered with a piece of 95% transparent acrylic plastic. The light under the other shelter—the high-quality shelter (HQ)—was covered with a piece of opaque black acrylic plastic to block the LED light and keep the shelter dark in daylight hours. Amblypygids are averse to bright lights (Santer and Hebets, 2009). Thus, if a subject were to sample LQ during an experimental session, they were expected to learn to avoid it.

Olfactory Cue

We provided a point source olfactory cue in the form of geraniol, a component of many essential oils (98% Geraniol, Sigma-Aldrich, Product Number 163333). A wide range of terrestrial arthropods is capable of detecting geraniol (Leonard and Masek, 2014; Hansson and Stensmyer, 2011), and *P. pseudoparvulus* is known to be capable of detecting monoterpenoids similar to geraniol (Hebets and Chapman, 2000). Because *P. marginemaculatus* are predators and do not directly rely on plants as a food source, we considered geraniol to be a relatively neutral source of olfactory information; that is, its presence was presumed to be neither particularly attractive nor aversive. The odor source was a 55 x 16-mm (diameter x height) plastic petri dish perforated with three 3-mm holes spaced 1-cm apart on one side, into which we inserted a 50-mm diameter circular filter paper (Whatman® qualitative filter paper, Grade 1**)** laden with 15-µL of geraniol. This design allowed the odor to slowly disperse into the air while restricting the animal from direct contact with the filter paper.

Lighting and Camera

Sessions were conducted on the same 12-h:12-h light:dark cycle in the same room that the animals were housed. The halogen lights attached to the arena walls and LED lights placed under the shelters were kept on a second timer that turned off these lights one hour before the overhead lights, and turned them on one hour after the overhead lights to simulate dawn and

dusk. The video tracker recorded the animals during these one-hour transitory periods between day and night, but tracking ceased once the arena lights turned on. The experimental room was kept completely dark at night except for two 9-watt red-filtered compact fluorescent lamps and high-power infrared LED flood lights that provided illumination to an infrared sensitive camera (Avemia Vari-focal CCTV Camera CMBB100) mounted above each arena and connected to a PC running our video tracking software. Both types of lamps produce a spectral range to which *P. marginemaculatus* are insensitive (Graving et al., In prep.). Therefore, there was no useful visual information available to subjects at night.

The Video Tracker

The Cartesian coordinates of the animal's position in the horizontal space of the arena were automatically extracted from each video frame using our own video tracking software or video tracking functions from the JavaGrinders library, a collection of freeware programming functions for the automated analysis of behavioral data (available at http://iEthology.com/). Both tracking algorithms are virtually identical and produced no noticeable differences in measured coordinates. Our tracking software was written in Python Version 2.7.1 (Python Software Foundation, available at http://www.python.org) using OpenCV Version 2.4.11 (Pulli et al., 2012). In brief, our tracking algorithm uses the background subtraction function cv2.BackgroundSubtractorMOG to separate each video frame into the foreground (the animal) and background (the arena). It then fits a contour to the outline of the segmented image of the animal using cv2.contours and calculates the Cartesian coordinates of the centroid using cv2.moments. As a subject moved around the arena, timestamps and coordinates of the animal's centroid were recorded once every 2-s and saved to a text file (kinematic variables such as linear

speed were then calculated post-hoc). The tracker was calibrated to track during nighttime hours and ceased tracking after the arena lights turned on.

Experimental Procedure

Several hours before a subject was placed into an arena for a session, we thoroughly cleaned the shelters and odor dishes with an unscented, aqueous detergent solution, rinsed them thoroughly with hot tap water, and allowed them to air dry. We also wiped the arena with 95% ethanol, allowed it to air dry, and ventilated the room until the ethanol odor dissipated. We selected the position of HQ for each session from one of four fixed positions (1-4) in the arena in a balanced randomized design such that each location was used a total of three times for the entire experiment (Fig.2C). We always placed LQ in the opposite (diagonal) corner. We placed the shelters in the arena and then randomly selected an animal, removed it from its home cage, and placed it in a clean plastic deli cup for several minutes until its movement had slowed and it was no longer startled. We then carefully transferred the animal to the arena, placing it directly inside HQ. We prepared an odor source, as previously described, and placed it in the corner next to HQ, perforated side facing the center of the arena (Fig. 2D-E). In the corner next to LQ we placed a petri dish that was prepared with 15-µL of reverse osmosis water, rather than geraniol. We then calibrated the infrared floodlights and camera to nighttime conditions and started the tracker. The start of a session was defined to coincide with the time that the overhead lights were turned off.

Each morning, we examined the tracking data, verified shelter choice and restarted the tracker. If a subject failed to exit a shelter on a particular night, we ran the session for an extra night so that each subject exited the shelter on at least five nights. We ended each session after the night that a subject exited the shelter for the fifth time. The session ended after five

consecutive nights for ten subjects and after six nights for 2 subjects. Eight of the 10 subjects that completed the session in five nights were held in the arena for an additional sixth night. No subject was kept in the arena for more than 7 nights. For formal analyses, we only used data from the first five nights during which a subject exited and returned to a shelter, and we provide choice data from the sixth night solely to demonstrate that successful homing behavior continues beyond the typical 5-night session. Subjects were fed the day before and immediately after the session but were not fed for the entire duration of the session.

Data Analysis

Coordinate data were plotted and checked visually for errors. Erroneous measurements were removed before further analysis. We rescaled coordinates from pixel units into real distance units (meters) and, for analytical and visualization purposes, we rotated the coordinate space so that HQ and LQ were in the same location for all subjects. We then calculated kinematic variables using custom scripts written in Python for further analysis.

Phenology and General Activity

We recorded several variables to characterize phenology and general activity, including the time interval between when the overhead lights turned off and when the animal exited the shelter (out-time), the interval between when the animal exited the shelter and returned to a shelter (activity-time), the interval between when the animal returned to a shelter and when the overhead lights turned on (in-time), and total activity (distance moved) over the course of the night (Table 1). We fitted linear mixed models (LMMs) to each variable separately with night number as a fixed effect with random slopes across nights and random intercepts by subject. We report time in units of hours and distance in units of meters.

Site Fidelity

To determine if *P. marginemaculatus* are capable of homing to a specific shelter we recorded shelter choice at the end of the night using the video tracking data, manually verified each morning. In three cases (one night for one subject, and two nights for a second subject), the coordinate data showed that an animal entered and remained in LQ before lights the overhead arena lights were turned on, but in the morning we found that the animal was in fact in HQ. These animals presumably switched shelters during daylight hours. In these cases, the trajectory data from the video tracker was used to designate the shelter choice as LQ. We analyzed shelter choice by fitting a binomial generalized linear mixed model (GLMM) including night number as a fixed effect with random slopes across nights and random intercepts by subject.

Space Utilization

We report on how individuals occupied the space of the arena as they moved throughout the night. The first variable we analyzed is *wall-following*. When introduced to a novel environment with limited visual cues, many species, including humans, exhibit wall-following behavior during which individuals maintain close proximity to a wall for an extended period of time. The function of this behavior may be related to spatial learning (reviewed in Patton et al., 2010). Here we define wall-following as the proportion of recorded points less than 5-cm from a wall. We chose this criterion after observing individuals touching the wall with their antenniform legs when in close proximity to a wall (described by Santer and Hebets, 2009). Here we assume 5 cm to be the approximate spatial limit of tactile perception for *P. marginemaculatus*. The maximum length from the distal tip of one antenniform leg to the center of the animal's body was less than 5 cm for all subjects. Thus, when a subject was less than 5 cm from the wall of the arena the walls of the arena may have guided the movement of the subject. In some species,

wall-following has been shown to decrease with time, a behavior which has been ascribed to the animal's ability to memorize its surroundings (Patton et al., 2010). We calculated the chance level for an animal to be found in this space as 0.19, the area of the outer 5 cm of the arena (0.19 m^2) divided by the total area of the arena (1 m^2).

We also recorded *shelter proximity* with HQ (P_{HQ}) and LQ (P_{LQ}), which we define as the proportion of recorded points in a night that a subject was on top of HQ or LQ or within 5 cm of a shelter wall (Table 1). This measure describes the proportion of time that animals are in close proximity to or are touching each shelter and provides further insight into whether or not individuals discriminate between shelters. One might anticipate that subjects would show an increase in the time spent near HQ and a decrease in the time spent near LQ over the course of a session if, as in natural conditions, animals tend to spend time near their home shelter (Weygoldt 2000). We calculated the chance level 0.038 for each shelter based on the area of a circle of radius 11-cm (the radius of the shelter, plus 5 cm) divided by the total area of the arena.

To analyze changes over the course of the session, we fitted LMMs to wall-following and shelter proximity. For wall-following, we included night number as a fixed effect with random slopes and intercepts by subject. For shelter proximity, we included night number, shelter (HQ or LQ) and the interaction between night number and shelter as fixed effects with random slopes across nights and random intercepts by shelter nested within subject. We arcsine square root transformed P_{HQ} and P_{LQ} to reduce heteroscedasticity before fitting the model.

Outbound and Inbound paths

We also report kinematic descriptions of the outbound and inbound paths subjects took as they exited and entered HQ throughout the trial. For these analyses, we used data only from nights where a subject both *exited and entered* HQ. The termination of an outbound path was

defined by the location of the subject when it first exceeded 0.84-m from the entrance of HQ (Table 1). The start of the inbound path was likewise defined as the last point during the night when a subject was farther than 0.84-m from the entrance of HQ. We chose 0.84 m as our criterion based on the distance between the shelter entrance and the furthest two walls (0.89 m). By using 0.84 m as our criterion we ensure that each animal was at least 5 cm from the walls opposite HQ at the start (inbound) or end (outbound) of the path. Additionally, this criterion optimizes the defined path so that the distance between start and end points of the paths is maximal while not artificially increasing the circuitousness of the path. We measured several variables for each inbound and outbound path including total path distance, mean distance from the wall, mean linear speed, and vector angle (Table 1).

Total path distance is the total distance the animal moved within each defined path and is utilized here as a measure of circuitousness. The straight-line distance between the start of the path and the shelter entrance is the same for all paths (0.84 m), so the greater the distance that a subject moves within the defined path the more circuitous is the path. Circuitousness provides a description of the directedness of the homing behavior; that is, a straighter path may indicate that the animal possesses knowledge of its goal location. Additionally, differences in path distance between outbound and inbound paths can provide insight into potential homing strategies, like path integration.

Mean distance from the wall was calculated as the average shortest distance between each recorded point and the nearest wall. This is another measure of wall-following behavior and provides us with insight into the use of the wall as a source of information during homing. One would expect individuals to remain in close proximity to the wall when using it as a source of information.

Mean linear speed was calculated as the average of the instantaneous linear speed recorded at each coordinate within each path. Like path distance, differences in linear speed between outbound and inbound paths provide insight into homing mechanisms, especially with regard to path integration.

We fitted LMMs to each variable to test for changes over time and differences between path types (outbound and inbound). For each model we included night number, path type and the interaction between night number and path type as fixed effects with random slopes across nights and random intercepts by path type nested within subject. To reduce heteroscedasticity, we log transformed mean distance from the wall and mean linear speed. For effect sizes, mean linear speed is reported in log[mm(s^{-1})], path distance is reported in meters, and mean distance from the wall is reported in log[cm].

Vector angles for outbound and inbound paths were calculated as the absolute angle of a line drawn between the end and start of the path and the entrance to HQ, respectively (Table 1). The confines of the arena limit the distribution of vector angles to values between -7.35° to 97.35° (shelter entrance defined as the origin of a unit circle). We use these angles not as a measure of homing accuracy, due to the physical restrictions of the arena, but instead as a measure of route fidelity in how an animal departs and returns to HQ. Whether a subject uses a (directionally) similar outbound and inbound path within a night is also of interest. To extract a value that describes this form of route fidelity, we calculated the mean vector from the average of the outbound and inbound vectors within each night for each subject, which we term the *route vector*. The outbound and inbound vector lengths for all individual paths equal 0.84 m so vector calculations on a unit circle with a radius of 1.0 is justified. To test for changes in the directional similarity of outbound and inbound paths across nights, we fitted a LMM to route vector length

that included night number as a fixed effect, random slopes across nights and random intercepts by subject.

To assess the consistency of outbound, inbound and route vectors at the population level, we calculated 95% confidence intervals for mean angle and mean vector length using multilevel bootstrap resampling (n = 10,000). We then compared these intervals to expected values for vector length and vector angle. The expected values of vector length and vector angle for all vector types were calculated by randomly sampling (n = 1,000,000) from a uniform distribution restricted to the range of possible vector angles within the arena (-7.35 to 97.35°). Expected values are approximately 0.87 for vector length and 45° for vector angle. Vector length values above 0.87 indicate high route fidelity while values below 0.87 indicate low route fidelity.

Finally, we visually inspected outbound and inbound paths for clues related to possible homing strategies. We provide plots along with qualitative descriptions of selected homing trajectories.

Statistical Analysis

We performed all statistical analyses with Python using the SciPy stack (Perez et al., 2011) or with R Version 3.1.2 (R Core Team, 2014). We used lme4 Version 1.1.7 (Bates et al., 2014) to create LMMs using lme4::lmer and GLMMs using lme4::glmer. All models were fitted using restricted maximum likelihood, and we report effect sizes as the mean ± standard deviation along with p-values calculated using normal approximation. To account for repeated measures, confidence intervals within figures were calculated using multilevel bootstrap resampling (n = 10,000), also referred to as “Case resampling”, with subject as the statistical unit, as implemented in the function seaborn.algorithms.bootstrap.

RESULTS

Phenology and General Activity

The activity patterns of subjects are summarized in Fig. 3. Individuals exhibited distinctive daytime and nighttime activity patterns. Daylight hours were spent in a shelter and subjects spent, on average, 7.53 ± 0.87 h of the 12-h dark period out of their shelters. On the first night subjects exited the high quality shelter on average 1.70 ± 0.90 h after the lights went out and their exit time from a shelter was consistently later as the session progressed, with out-time increasing 0.08 ± 0.04 h per night ($z = 2.00$, $p = 0.047$). Subjects returned to their shelter on average 2.96 ± 0.63-h before the overhead lights came on, but returned earlier as the session progressed, with in-time increasing 0.55 ± 0.21 h per night ($z = 2.71$, $p = 0.007$). Thus, total nocturnal activity time decreased -0.64 ± 0.23 h per night over the session ($z = -2.82$, $p = 0.005$). The reduced activity period over the session corresponded to a reduction in the distance traversed by subjects in the arena. Individuals moved on average 112.42 ± 24.06 m per night, but movement decreased -20.60 ± 3.50 m per night over the session ($z = -5.876$, $p < 0.001$).

Site Fidelity

P. marginemaculatus reliably homed and learned to discriminate between shelters. Animals returned to HQ more often as the session progressed (Fig. 4). The probability of return started near chance levels on the first night and significantly increased ($z = 2.33$, $p = 0.020$) on each successive night, reaching perfect site fidelity (1.0) for all animals by the fifth night. Half of the 12 subjects showed perfect fidelity to HQ; that is, these animals never chose LQ. This clearly illustrates that *P. marginemaculatus* not only discriminate between shelters *after* sampling LQ, but also exhibit high site fidelity *without* sampling LQ.

Space Utilization

Wall-following was consistently high and did not change over the course of the session ($z = 0.78$, $p = 0.43$). The proportion of points within 5 cm of the wall was greater than chance levels on all nights, with individuals spending more than half of their activity time in proximity to the wall (Fig. 5). Individuals spent more time around HQ and a relatively consistent amount of time around LQ as the session progressed (Fig. 6). The difference ($P_{HQ} - P_{LQ}$) increased across nights ($z = 2.33$, $p = 0.019$).

Outbound and Inbound Paths

Results of kinematic comparisons between inbound and outbound paths are given in Table 2. Only one variable differed between the routes—distance from the wall—but the magnitude by which this variable differed between paths changed across nights. Overall, outbound paths tended to be farther from the wall than inbound paths and, while outbound paths remained consistently far from the wall, inbound paths became closer to the wall with each night as the session progressed (Fig. 7). Mean linear speed declined across nights but did not differ between path types (Fig. 8). Path distance (circuitousness) did not change across nights or differ between inbound and outbound paths (Fig. 9).

Route vector lengths were around the chance expectation and did not change across nights (Fig. 10). The angular characteristics of inbound and outbound paths are shown in Figure 11. Vector length confidence intervals for outbound, inbound, and route vectors were all below the expected value of 0.87 indicating low outbound and inbound route fidelity across nights and directional inconsistency between outbound and inbound paths. Vector angle confidence intervals for outbound, inbound, and route vectors all overlapped the chance expectation of 45^{o},

which implies that animals followed the wall but did not exhibit fidelity in their choice of wall. Plots of typical outbound and inbound paths are shown for three selected subjects in Figure 12.

Figure 13 shows three notable return routes, which implicate odor as a discrimination cue between HQ and LQ. Subjects were observed successively walking on top of both petri dishes before entering HQ (Fig. 13A). In multiple cases, subjects explored the area around LQ before cutting across the center of the arena to return to HQ (Fig. 13B). One subject repeatedly showed an expanding spiral inbound path, typical of olfactory-guided homing, before walking to the odor source near HQ and then subsequently entering HQ (Fig. 13C).

DISCUSSION

Homing and Activity in P. marginemaculatus

The results of this study clearly illustrate that *P. marginemaculatus* reliably exit and return to a shelter each night and that they are capable of homing in the laboratory. The preference for HQ over LQ, which increased over nights of the session, implies that subjects learned to discriminate between the two shelters. Indeed, subjects spent more time in proximity to HQ as the session progressed, which suggests that they recognized HQ. The general activity patterns of *P. marginemaculatus* that we observed seem to be consistent with that of a typical nocturnal animal. The greatest amount of activity occurred in the middle of the night rather than during twilight hours (crepuscular) or during the day (diurnal). The observed changes in phenology patterns and activity levels over the course the session also suggest a learned familiarity with the arena. Overall, animals exited their shelters later, returned earlier, spent less time in the arena, and exhibited lower activity levels as the session progressed. This suggests a decrease in exploratory behavior, typically induced by novel environments, and perhaps increased familiarity with the spatial components of the arena (Mikheev and Andreev, 1993; Teyke, 1989). Of course, because we did not feed the animals for the duration of the session, this observed change of behavior could be a result of fatigue (or a combination of fatigue and learning). Further experimentation is required to ascertain this possibility.

Sensory Modalities Used for Homing

P. marginemaculatus relied on sensory modalities other than vision for orientation while homing. All subjects used in this study were deprived of visual cues, so we can eliminate this modality as being necessary for successful homing. This is consistent with most other nocturnal arachnids, where vision typically plays a lesser role or no role at all (Foelix, 1996) except in a

few notable cases (Norgaard et al., 2008, Dacke et al., 1999). Vision may, however, still contribute to homing. Indeed, we are in the process of studying the visual system of *P. marginemaculatus* in more detail (Graving et al., In prep.). Additionally, because the only salient cue we provided during the experiment was a point-source chemical odor, we conclude that *P. marginemaculatus* likely used olfactory information to discriminate between shelters. As illustrated in Figure 13A, we observed individuals sequentially sampling the area around both odor dishes (located in opposite corners in the arena) before choosing HQ, suggesting that *P. marginemaculatus* are capable of discriminating spatial locations based on olfactory information. We also observed multiple subjects rapidly cutting across the center of the arena before returning home (Fig. 13B), which, although anecdotal, could be ascribed to a beaconing strategy (i.e., orienting to a distant, salient landmark). Another subject performed what appeared to be a type of spiral search (Fig. 13C), similar to olfactory-guided behavior observed in other animals (Calhoun et al., 2014; Svensson et al., 2014). We hypothesize that odor sources themselves served as local landmarks for homing while the odor plumes these landmarks produced may have been exploited for use in a beaconing strategy. It has been hypothesized that many animals use sources of olfactory information for homing and have evolved brain centers for exploiting olfactory gradients for use in spatial tasks (Svensson et al, 2014; Jacobs, 2012). Indeed, we know that, relative to their body size, amblypygids possess massive mushroom bodies, an invertebrate brain region associated with olfactory learning, memory, and multisensory integration (Wolff and Strausfeld, 2015; Strausfeld et al., 1998). This brain region contains several million neurons, and variations in its size and complexity across arthropod taxa has been ascribed to learning and navigation abilities (Wolff and Strausfeld, 2015; Strausfeld, 2012, pp. 371, 554-555; Jacobs, 2012). Navigation and orientation behavior in odor plumes has been

investigated in a variety of animals, including arthropods (reviewed in Svensson et al., 2014; Vickers, 2000), and the results of this study strongly suggest that *P. marginemaculatus* are similarly able to use olfactory information for homing.

The Wall as a Source of Information

In this study, subjects appear to have used the wall as a source of information for homing. Individuals consistently remained in close proximity to the wall for more than half of the time they spent outside a shelter. Moreover, inbound paths were in closer proximity to the wall than outbound paths and became closer to the wall on average with each night. The results of our vector analyses also demonstrate that individuals exhibited low route fidelity when exiting and returning to HQ and typically departed and approached HQ along the walls of the arena. Outbound, inbound and route vector lengths were all significantly lower than the expected random value (0.87) while outbound, inbound, and route vector angles were not significantly different from the expected random value (45°). Route vector lengths also did not significantly change over the course of the session. Together these results suggest that individuals did not exhibit route fidelity when departing or approaching HQ and were not approaching and departing HQ in random directions. Instead, these results reveal that individuals tended to depart and return to HQ along the wall, but were not consistent in their choice of wall within or across nights. Indeed, the increased proximity to the wall we observed across a session for inbound paths (and not outbound paths) suggests subjects may have learned that the odor landmark, an indication of proximity to HQ, was located near the wall. With the walls as a guide, the odor dishes and shelters likely provided sufficient landmarks by which *P. marginemaculatus* could home to HQ. The robust wall-following behavior observed in this study was likely a product of the location of the shelters and odor dishes. Indeed, each of these potential landmarks in the arena was placed

near a wall, and this would make wall following especially advantageous for a landmark-based homing strategy. The use of local landmarks for homing has been demonstrated in at least one other species of arachnid (Nörgaard et al., 2007) and is a common strategy for homing in other arthropods (Dyer, 1998; Papi, 1992).

Behavioral Strategies in Amblypygid Homing

Field displacement studies showed that *P. pseudoparvulus* does not rely on path integration to home (Hebets et al. 2014a). The kinematic similarities we observed between the outbound and inbound paths in this study suggest that path integration is also unnecessary for homing by *P. marginemaculatus*. In most cases of path integration, outbound and inbound paths differ substantially in their movement characteristics (Kamran and Moore, 2015; Etienne and Jeffrey, 2004; Wallace and Whishaw, 2003; Wallace et al. 2002; Müller and Wehner, 1994; Wehner and Wehner 1990; Müller and Wehner, 1988; Seyfarth et al. 1982; Seyfarth and Barth 1972). Specifically, Wallace et al. (2006) found in laboratory rats that outbound paths are highly circuitous and slow moving, while inbound paths are highly goal-directed and fast moving. Kamran and Moore (2015) also found a comparable pattern in two species of crayfish, suggesting that these movement characteristics are common across taxa. We found no differences between outbound and inbound path length (circuitousness) and mean linear speed, indicating that behavioral strategies other than path integration may be the norm for this species. One major difference between this study and those conducted by Hebets et al. (2014a,b) is the method by which homing behavior was initiated. In the case of Hebets et al. (2014a,b), *P. pseudoparvulus* individuals were manually displaced several meters, while in this study *P. marginemaculatus* individuals were allowed to freely move around a space that is only 1-m x 1-m. The displacement method used by Hebets et al. (2014a,b) could have forced individuals to

rely on some mechanism other than path integration, as information available to subjects about the distance and angle of their displacement was presumably limited. However, our study seems to confirm that amblypygids do not explicitly rely on path integration regardless of whether or not the animal is displaced.

P. marginemaculatus as a Model Organism

P. marginemaculatus may be ideal for studying the sensory, cognitive, and behavioral mechanisms by which amblypygids home. Here we have established a framework for studying amblypgyid homing in the laboratory under tightly controlled conditions and provided insight into the types of information and behavioral strategies amblypygids may exploit for successfully returning to a shelter at night. Although these results suggest vision is unnecessary for homing, our recent characterization of the visual system in *P. marginemaculatus* indicates that this species, and other amblypygid species, may be capable of exploiting visual information as an orientation cue (Graving et al, In prep.). Further research should focus on amblypgyid homing in both unimodal and multimodal contexts. We hypothesize that not only are amblypygids capable of using multimodal information, but multimodal strategies may in fact be necessary for successful homing in their natural, challenging nocturnal environments.

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APPENDIX A: TABLE 1

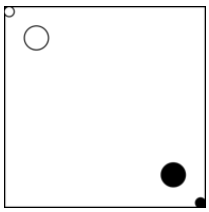
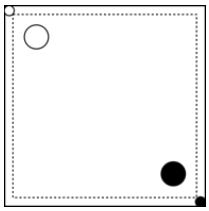
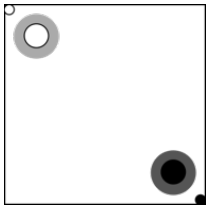
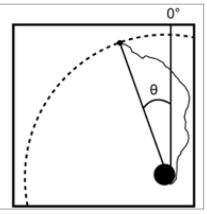
Analysis	Variable	Description	Figure
Site fidelity	shelter choice	The binary choice (HQ = 1, LQ = 0) for each subject on each night. Chance level = 0.50	
Phenology and activity	out-time (hours)	The time interval between lights off and when the animal exited the shelter	
	in-time (hours)	The time interval between when the animal returned to a shelter and lights on	
	activity-time (hours)	The time interval between when the animal exited a shelter and returned to a shelter at the end of the night	
	total activity (m)	The total distance moved over the course of a night	
Space utilization	wall-following	Proportion of points recorded within 5-cm of a wall. Chance level is the outer 5-cm area divided by the total area of the arena, or 0.19.	
	shelter proximity	Proportion of points recorded at each shelter and within 5-cm of a shelter wall. Chance level is the area of each circle divided by the total area of the arena, or 0.038 for each shelter.	
Path kinematics	path distance (m)	Total distance of the defined path.	
	vector angle (°)	Angle of line (θ) from end point (outbound) or start point (inbound) to the entrance of HQ.	
	mean distance wall (cm)	Averaged distance to each point from the nearest wall for each path.	
	mean linear speed (mm•s^{-1})	Averaged linear speed (distance•time^{-1}) of each point within each path	

Table 1: A description of each variable separated by analysis type. Figures illustrate how site fidelity, wall-following, shelter proximity and vector angle were calculated in the space of the arena. Filled circles represent HQ and the odor dish while open circles represent LQ and the water dish. The dashed bounding box indicates the criterion we used for calculating wall-following. The gray rings around HQ and LQ indicates the criterion we used to calculate shelter proximity. The dashed curvilinear line represents the criterion we used for defining outbound and inbound paths, while the solid lines illustrate how we calculated the vector angle for each trajectory.

APPENDIX B: TABLE 2

Response	Fixed Effects	Estimate	Std. Error	*z*	P > | *z* |
path distance (m)	(Intercept)	1.633	0.345	4.733	<0.001
	Night	0.061	0.115	0.526	0.599
	Path	-0.106	0.471	-0.225	0.822
	Night x Path	-0.101	0.154	-0.653	0.523
log[mean wall distance (cm)]	(Intercept)	1.747	0.129	13.500	<0.001
	Night	-0.003	0.046	-0.0766	0.939
	Path	**0.073**	**0.023**	**3.168**	**0.002**
	Night x Path	**0.049**	**0.007**	**7.373**	**<0.001**
log[mean linear speed (mm·s^{-1})]	(Intercept)	1.843	0.128	14.432	<0.001
	Night	**-0.093**	**0.040**	**-2.318**	**0.020**
	Path	0.129	0.167	0.774	0.438
	Night x Path	-0.040	0.050	-0.800	0.423
route vector length	(Intercept)	0.937	0.045	20.834	<0.001
	Night	-0.027	0.017	-1.638	0.101

Table 2: LMM results for path kinematics (bold indicates significance)

APPENDIX C: FIGURE 1

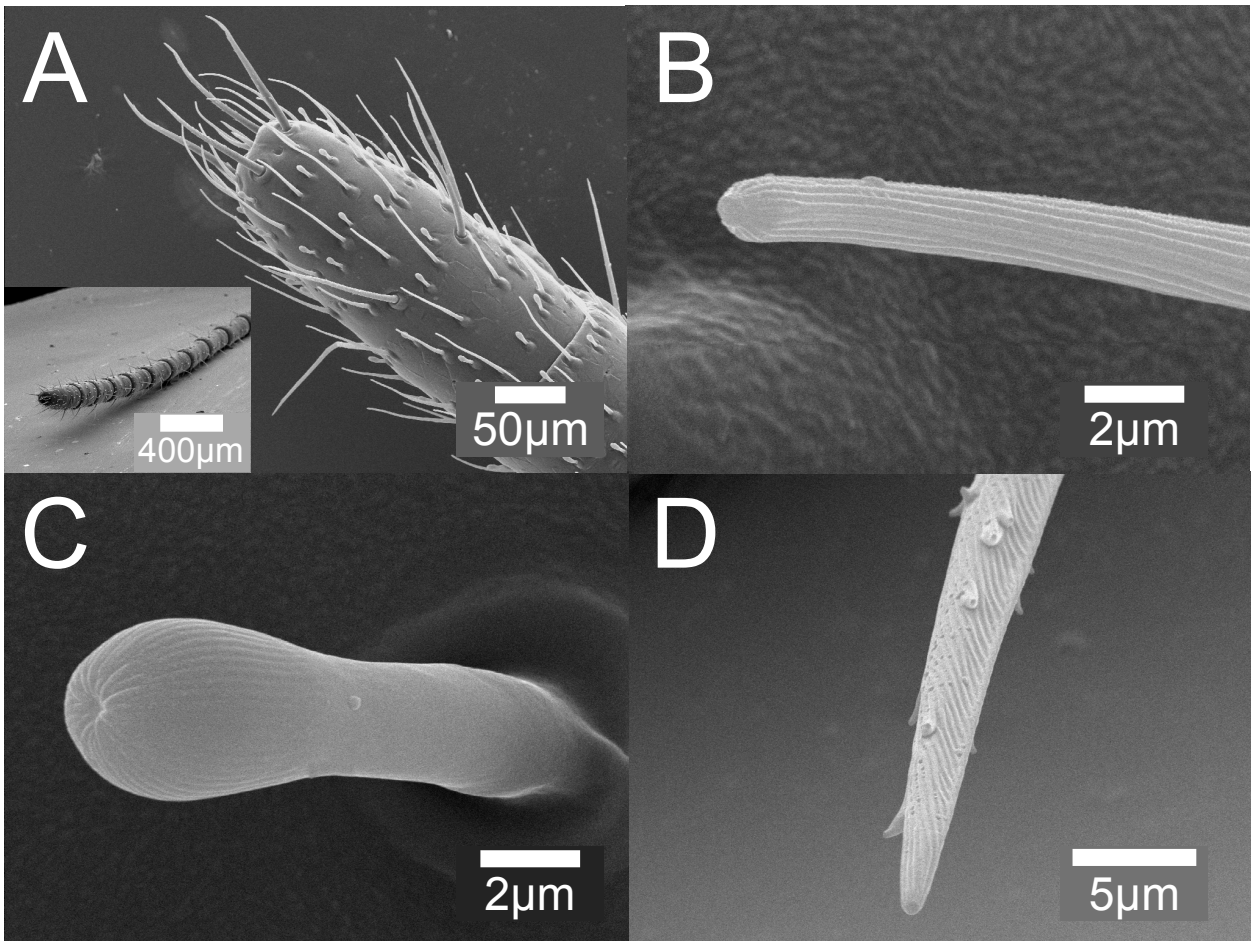


Figure 1: Sensory hairs of the antenniform legs. Electron micrographs of (A) the distal tip of an antenniform leg (inset). (B,C,D) Sensory hairs are densest at the distal end of the antenniform leg with chemosensory hairs known as (B) porous sensilla and (C) club sensilla not present beyond 25 segments from the tip. This leaves only mechanosensory hairs known as (D) bristle sensilla on the most proximal segments of the antenniform legs.

APPENDIX D: FIGURE 2

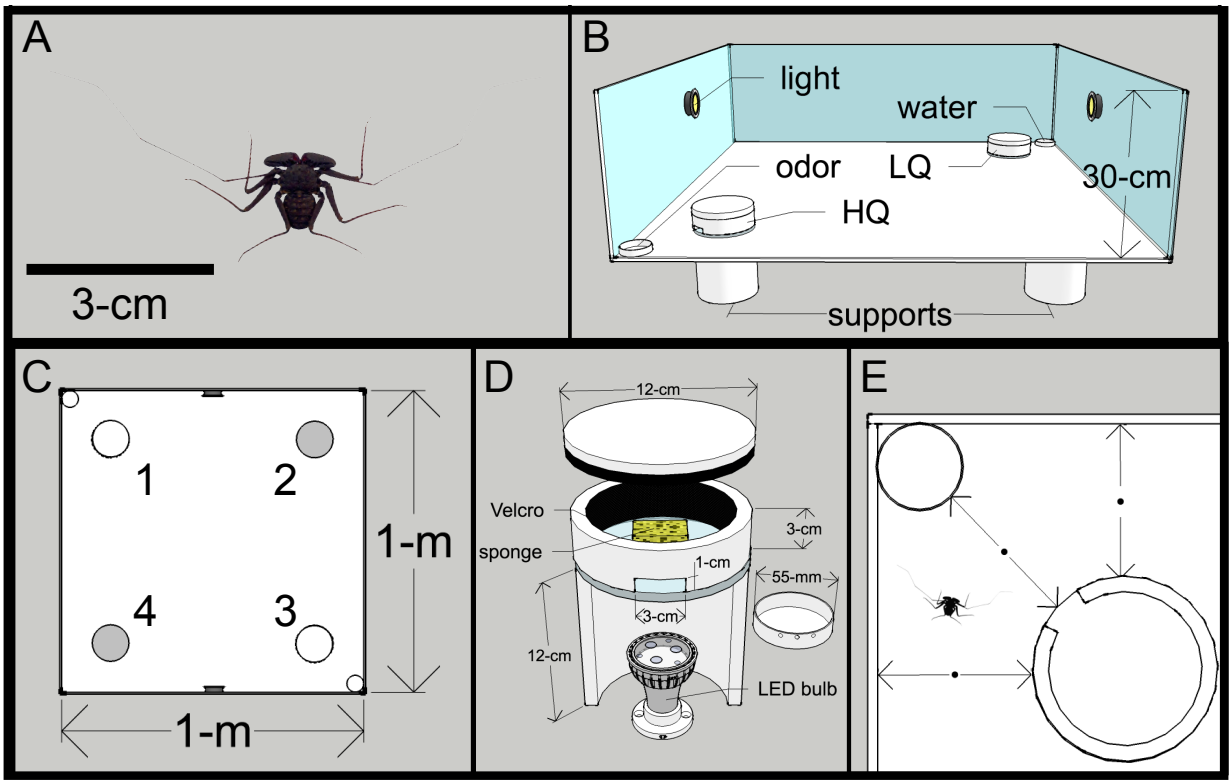


Figure 2: The experimental setup. (A) A close-up view of *P. marginemaculatu*s. (B) The general layout of the arena during a session. (C) A top down view of the arena illustrates the four possible shelter positions (1-4) with shelters always placed diagonally opposite from each other (gray and white circles illustrate possible combinations). (D) A detailed view of the shelter, odor cue, the support (under shelter) and the odor source (small dish on right). LED output was covered with either clear plastic (LQ) or opaque, black plastic (HQ). (E) A detailed top down view of the shelter and odor cue positioning with *P. marginemaculatus* shown to scale; all arrows indicate a distance of 10-cm.

APPENDIX E: FIGURE 3

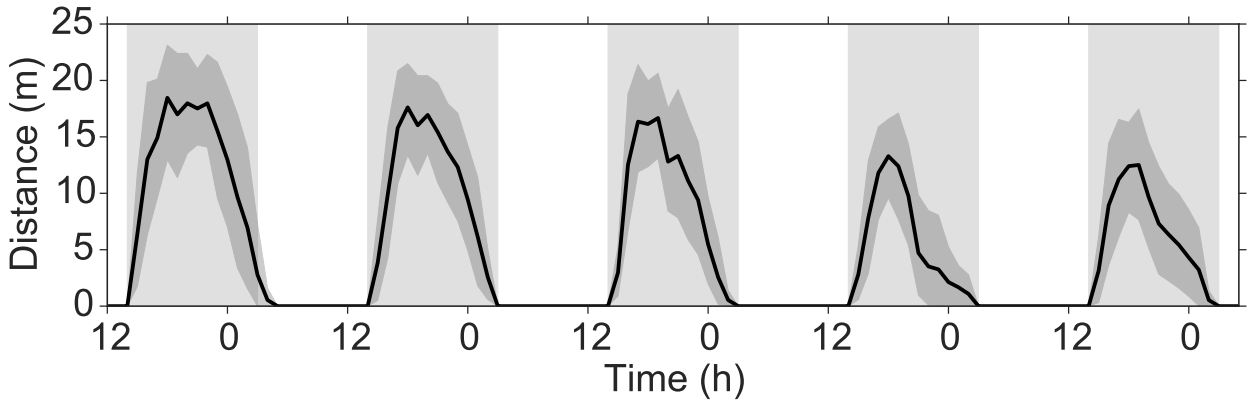


Figure 3: Nocturnal activity in the arena. Gray bars indicate the time interval when the overhead lights were turned off. *P. marginemaculatus* exhibited activity patterns consistent with a nocturnal animal. Black lines show the mean (± 95% CI) for distance moved, binned for each hour for all animals (n = 12). Activity is highest in the middle of the night with less activity toward the beginning and end of the night.

APPENDIX F: FIGURE 4

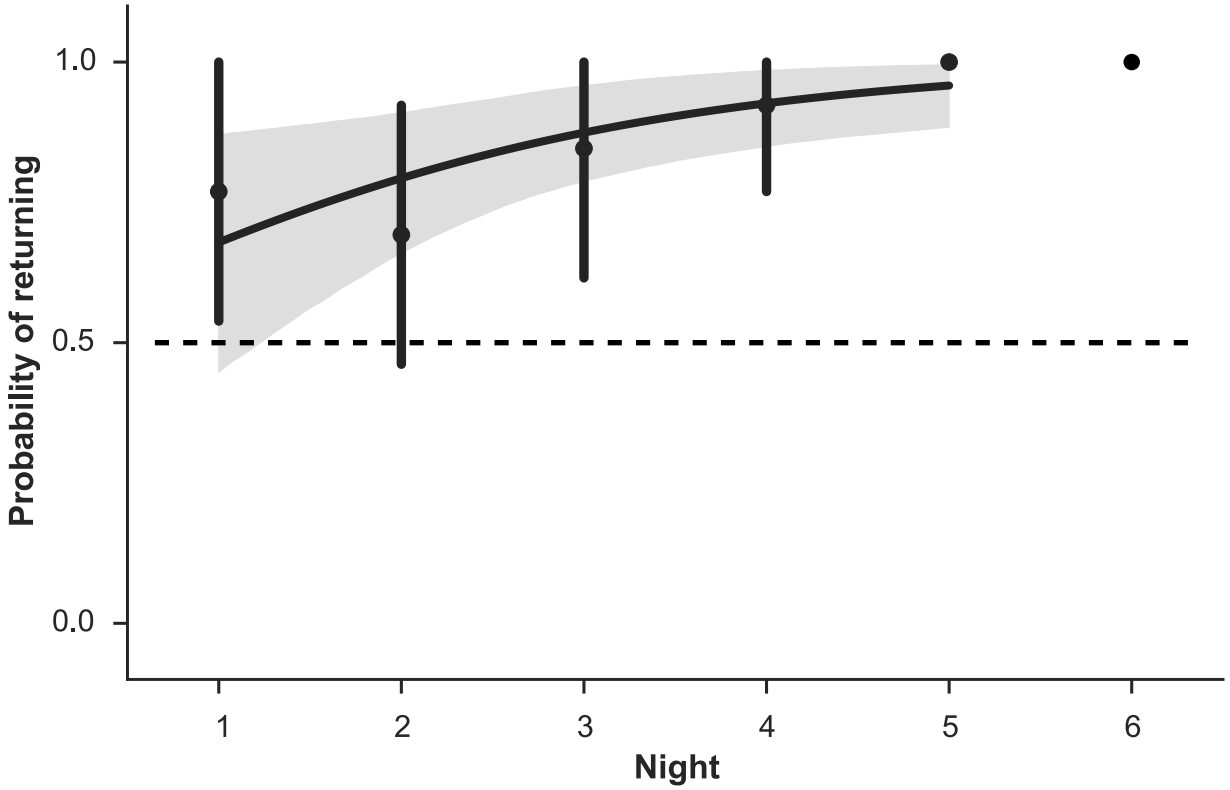


Figure 4: Site fidelity. Dots show the mean probability (±95% CI) of returning to HQ for each night, and the solid line shows the binomial GLMM fitted to the data. The dashed line indicates chance level (0.50). The first 5 nights of data were used to fit the model (n = 12). Data from night 6 (n = 8) are shown only to demonstrate the robustness of site fidelity after the initial 5-night session.

APPENDIX G: FIGURE 5

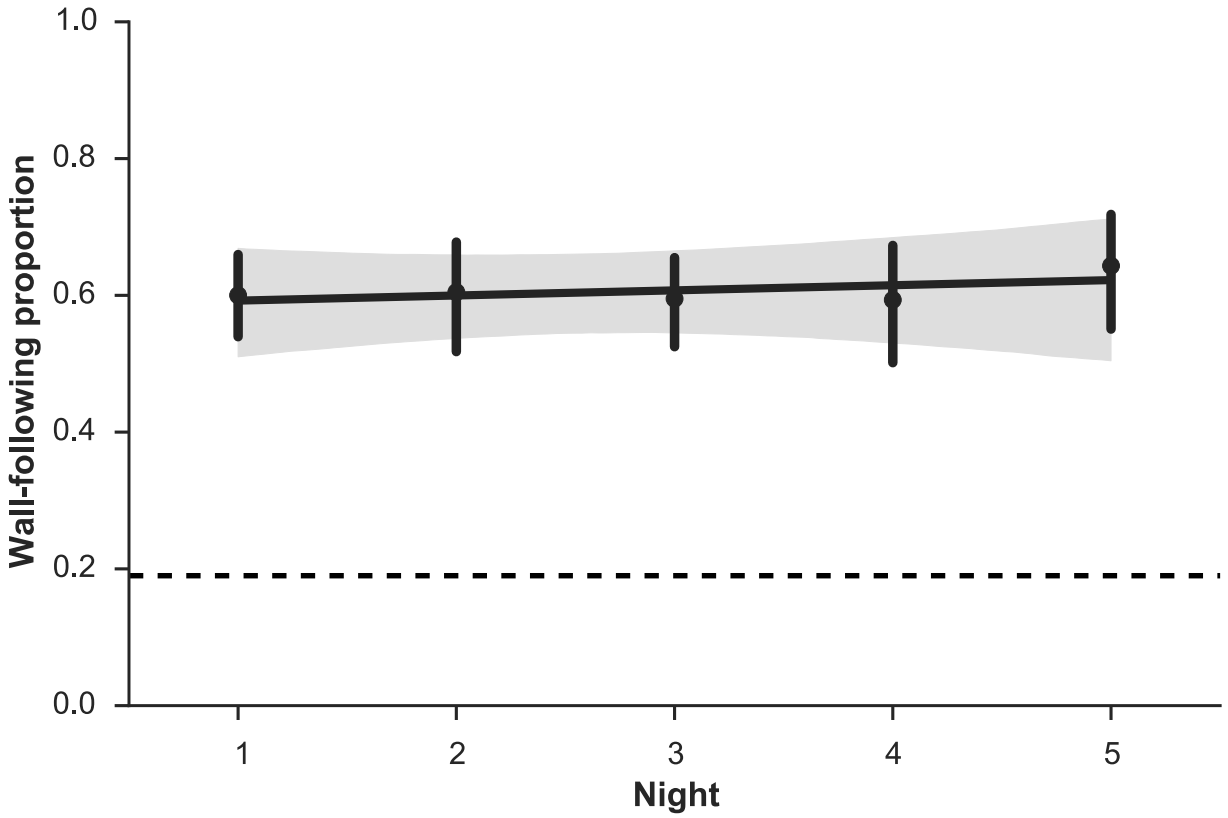


Figure 5: Wall following. Dots show the mean proportion (± 95% CI) and the solid line shows the line fitted to the data. The proportion of points in the outer 5-cm of the arena was consistently higher than chance levels (0.18; dashed line) but did not change across nights.

APPENDIX H: FIGURE 6

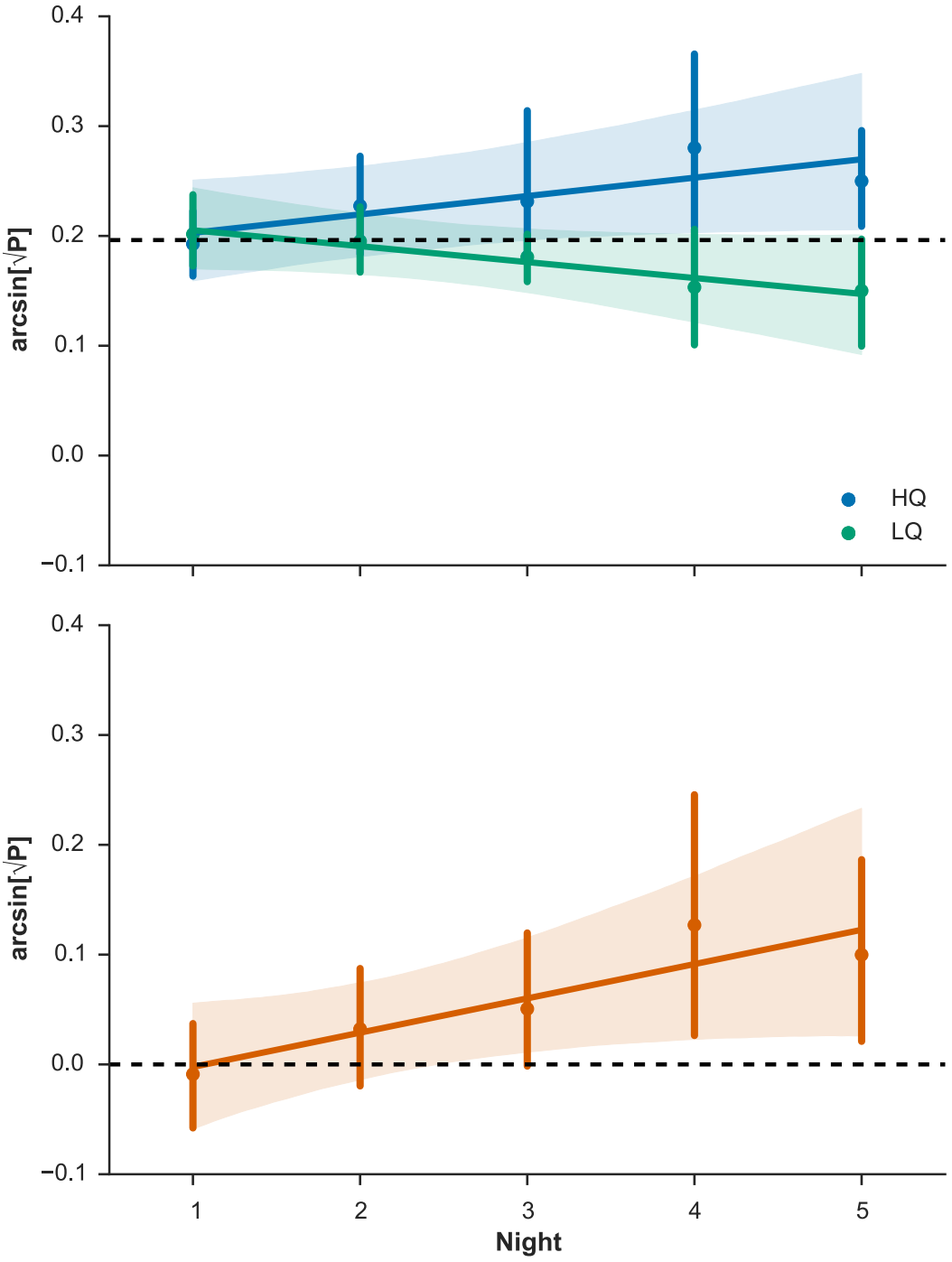


Figure 6: Shelter proximity by subjects across nights. (Top) Dots show the mean proportion (±95% CI) of points recorded on and around each shelter across nights. (Bottom) Dots show mean pairwise differences (±95% CI) between shelters for each night. The corresponding solid lines (± 95% confidence bands) show the estimated effect of time (nights) from the LMM. HQ and LQ start at chance levels (0.19; dashed line) and diverge as the session progresses with HQ increasing above chance.

APPENDIX I: FIGURE 7

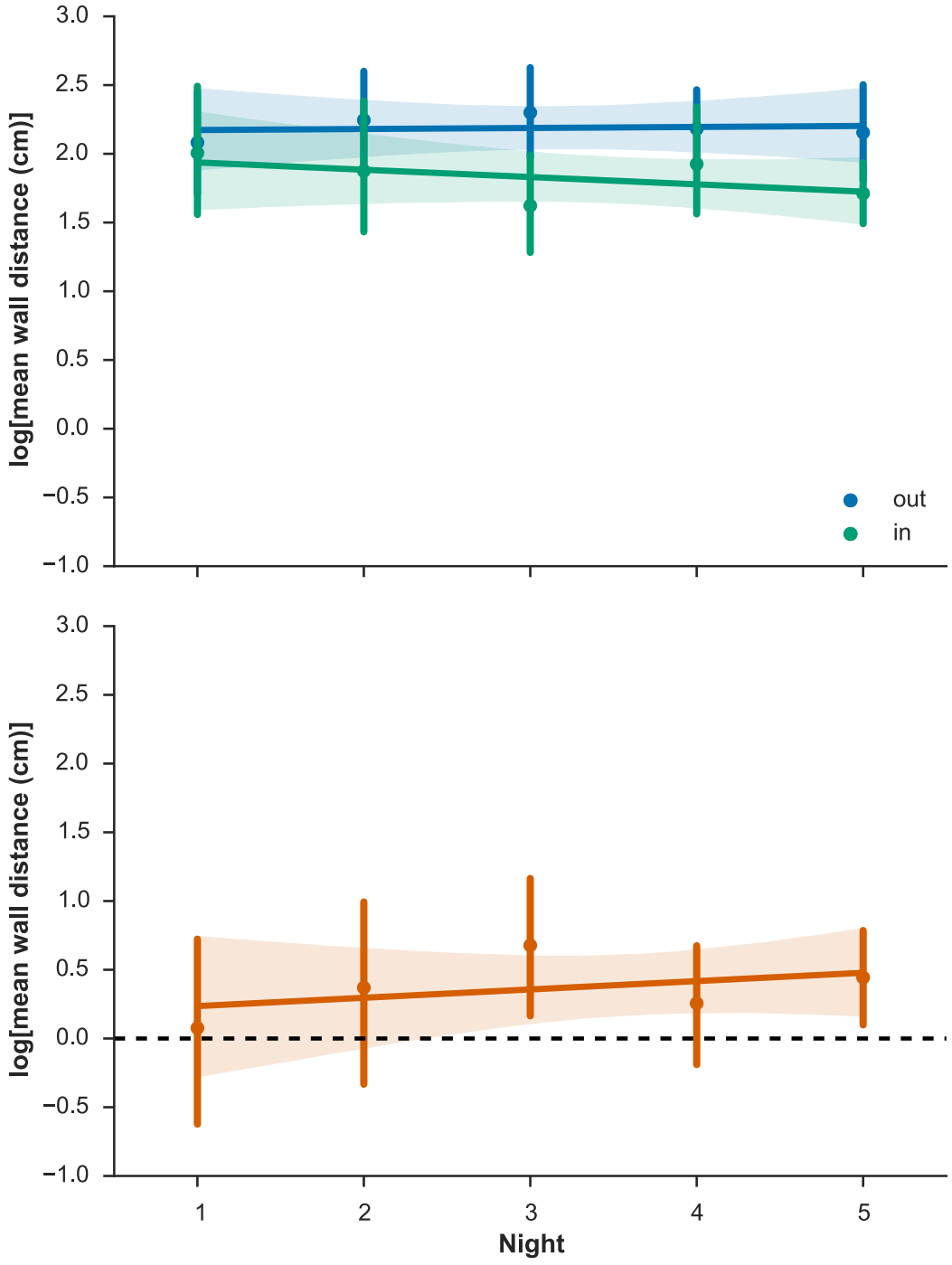


Figure 7: Log[mean wall distance] for outbound and inbound paths. (Top) Dots show the mean values (±95% CI) for each path type across nights. (Bottom) Dots show mean pairwise differences (±95% CI) between path types for each night. The corresponding solid lines (± 95% confidence bands) show the estimated effect of time (nights) from the LMM.

APPENDIX J: FIGURE 8

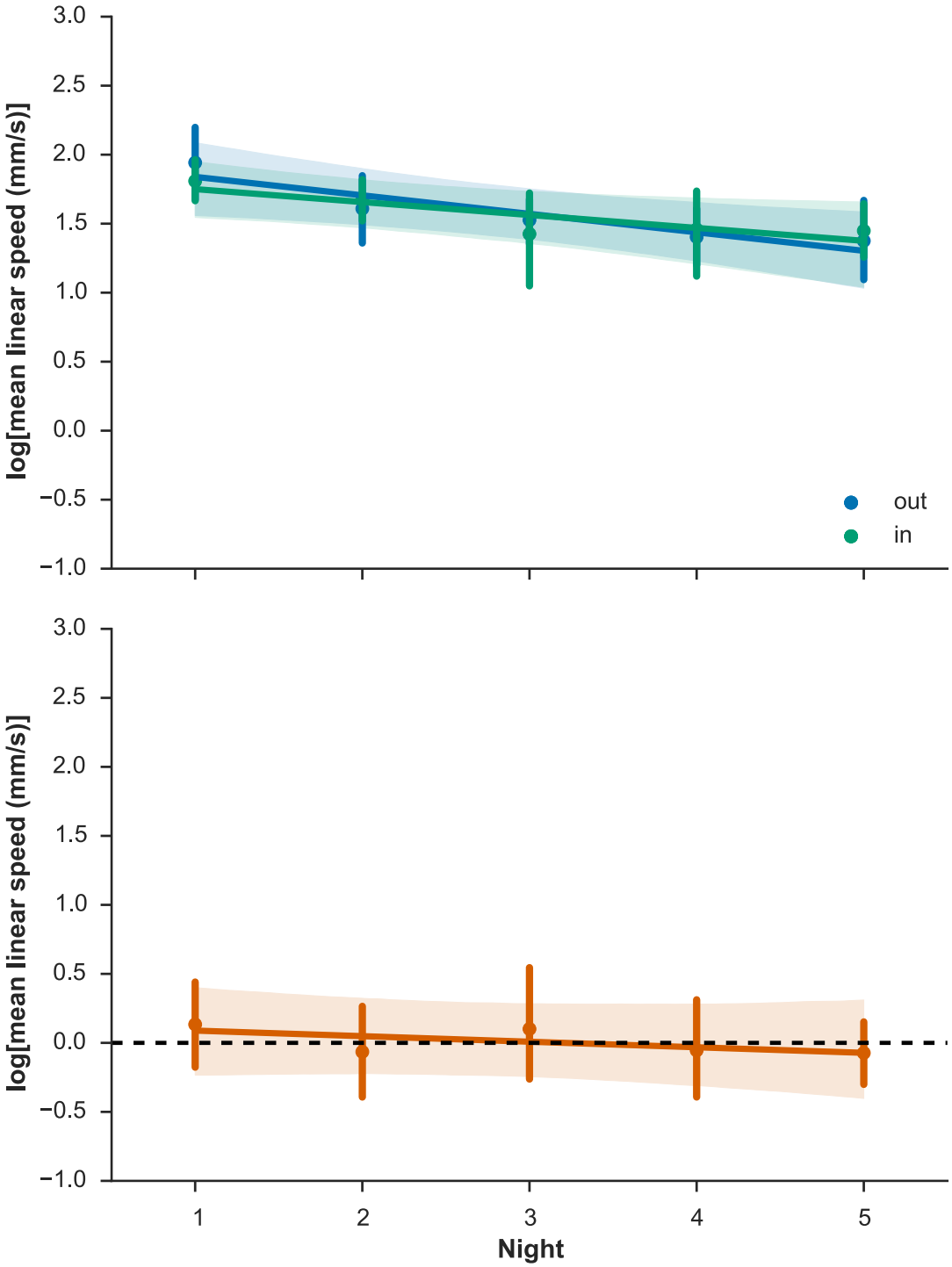


Figure 8: Log[mean linear speed] for outbound and inbound paths. (Top) Dots show the mean values (±95% CI) for each path type across nights. (Bottom) Dots show mean pairwise differences (±95% CI) between path types for each night. The corresponding solid lines (± 95% confidence bands) show the estimated effect of time (nights) from the LMM.

APPENDIX K: FIGURE 9

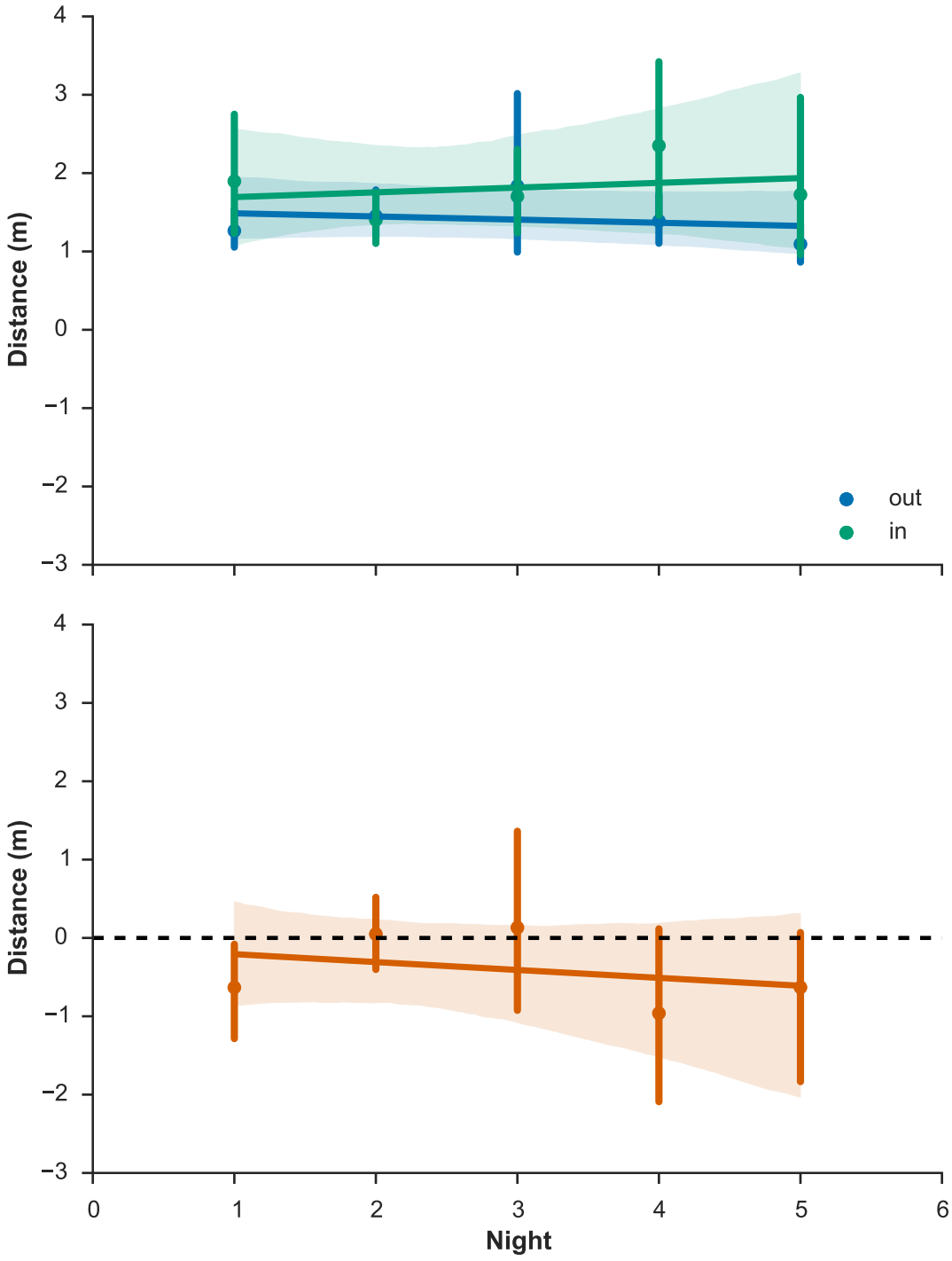


Figure 9: Path distance (circuitousness) for outbound and inbound paths. (Top) Dots show the mean values (±95% CI) for each path type across nights. (Bottom) Dots show mean pairwise differences (±95% CI) between path types for each night. The corresponding solid lines (± 95% confidence bands) show the estimated effect of time (nights) from the LMM.

APPENDIX L: FIGURE 10

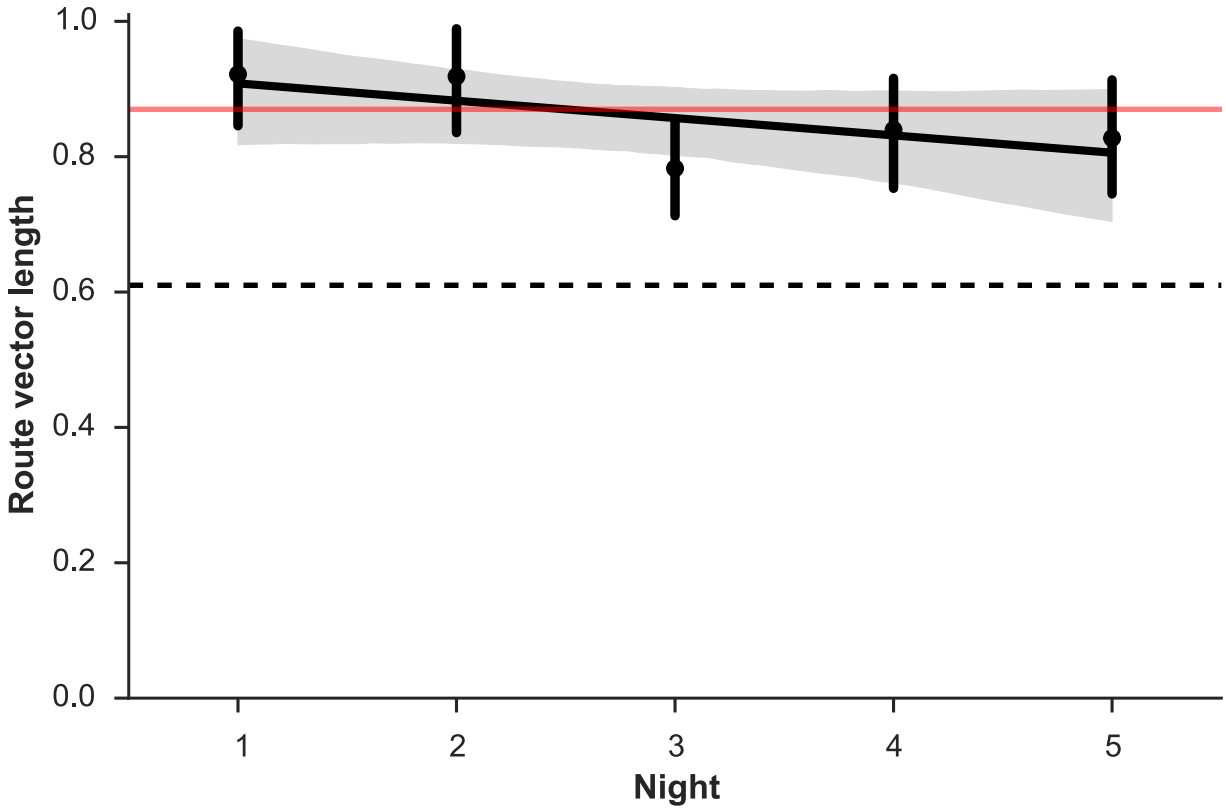


Figure 10: Route vector lengths. Dots show the mean vector length (± 95% CI) across nights. The corresponding solid line (± 95% confidence bands) shows the estimated effect of time (nights) from the LMM. Route vector lengths did not significantly change across the session. The dashed line indicates the minimum possible vector length (0.61). The red line indicates the expected random value (0.87).

APPENDIX M: FIGURE 11

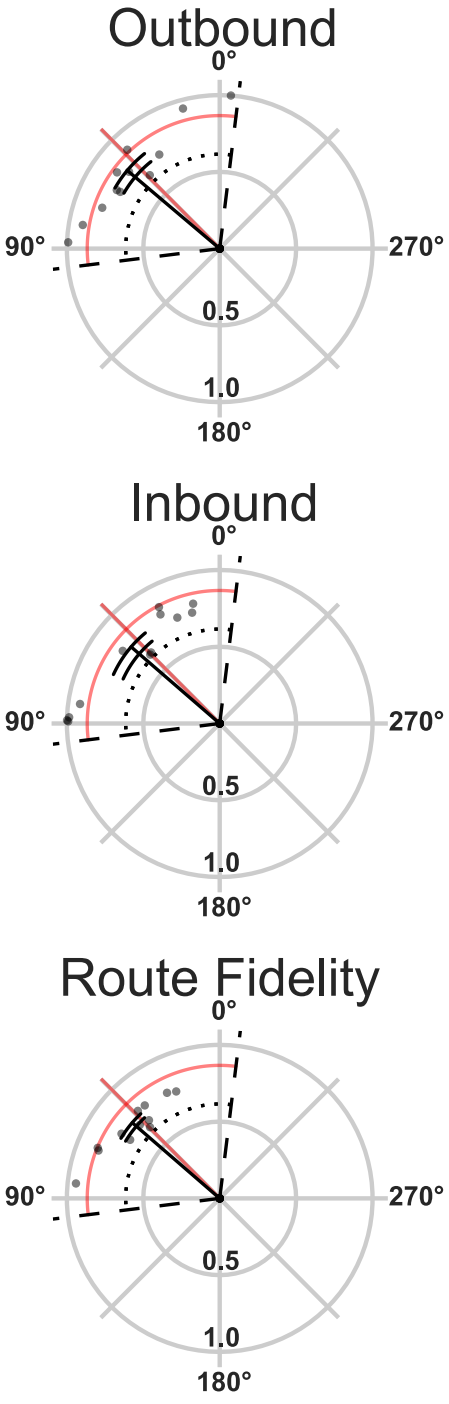


Figure 11: Mean vectors (± 95% CI) for outbound, inbound, and route vectors. Solid black arcs indicate 95% CI for mean vector angle and the interval between them indicates the 95% CI for mean vector length. Red lines indicate expected values for mean vector angle (45°) and mean vector length (0.87). Dashed lines indicate the possible distribution of angles (-7.35 to 97.35°), and dotted arcs indicate the minimum possible mean vector length (0.61).

APPENDIX N: FIGURE 12

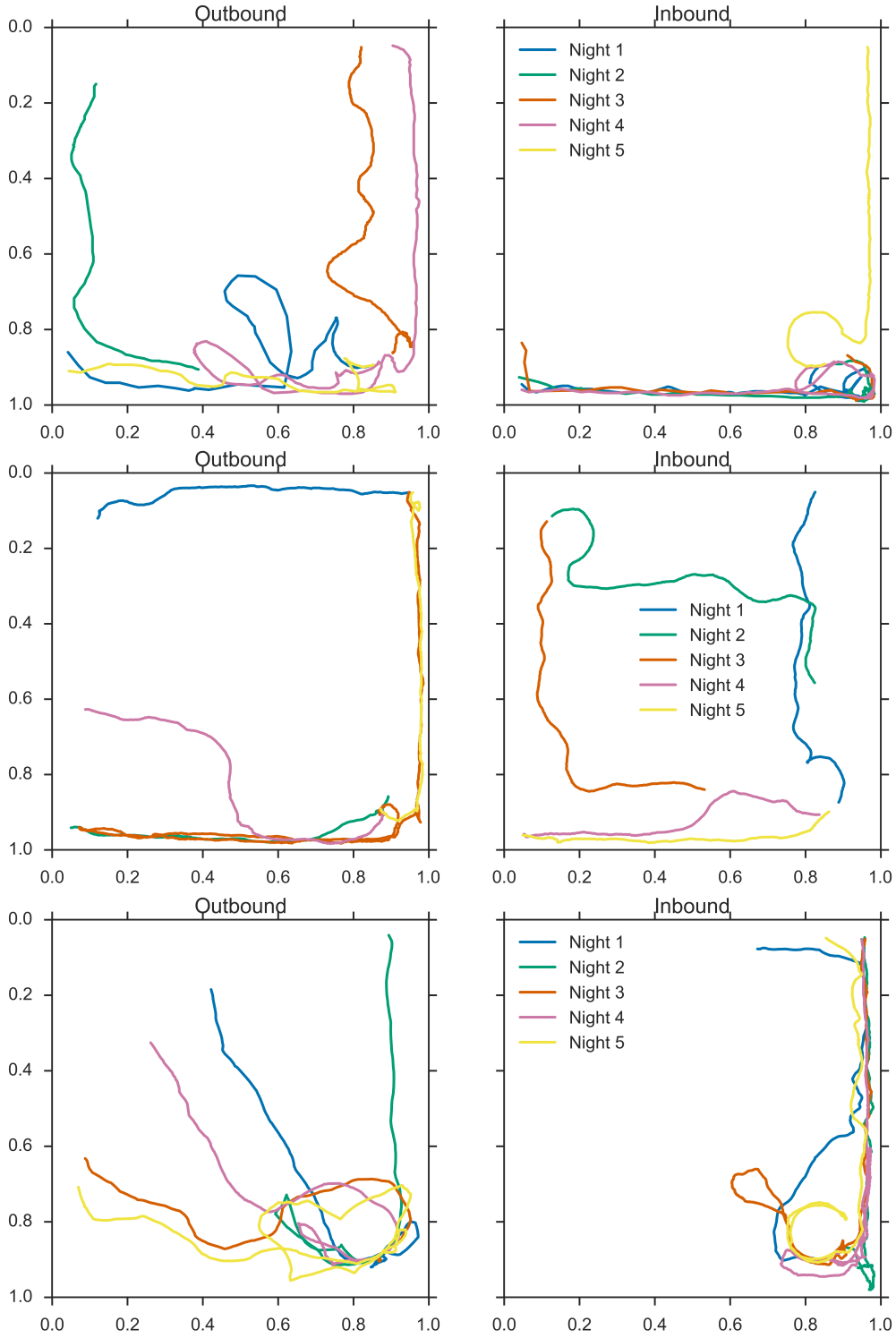


Figure 12: Outbound and inbound paths for three subjects. Axes correspond to arena walls. LQ is located in the top left corner, while HQ is located in the bottom right corner.

APPENDIX O: FIGURE 13

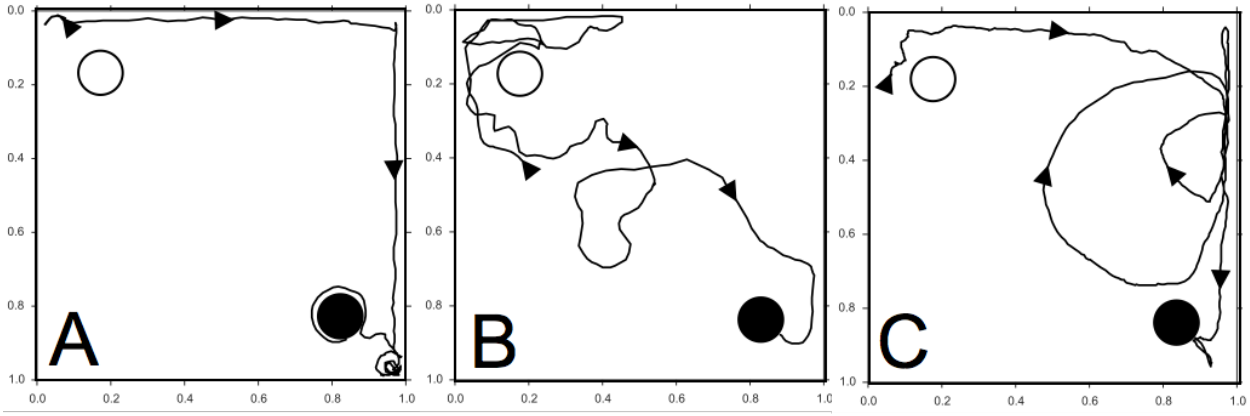


Figure 13: Notable inbound paths for selected subjects. These inbound trajectories suggest olfactory cues are vital for successful homing in *P. marginemaculatus*.